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THE BOTANICAL REVIEW

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PRODUCTION OF VITAMIN B_{12} BY MICROORGANISMS AND ITS OCCURRENCE IN PLANT TISSUES[1]

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INTRODUCTION

Interest in vitamin B_{12} had its origin in 1926 when Minot and Murphy (84) discovered the effectiveness of liver therapy for pernicious anemia. Since that time, a considerable number of relatively highly concentrated liver preparations have been developed by chemists and have been used successfully in clinical treatment. These developments were thoroughly reviewed in 1945 by Subbarow (137) and will not be discussed here.

In 1948 the anti-anemia factor present in liver was isolated in crystalline form. In that year independent investigators in the United States and in England almost simultaneously reported the isolation of vitamin B_{12}, one of the most potent physiologically active substances known. Rickes et al. (102) announced the isolation of biologically active crystalline material from liver concentrates, and Smith and Parker (129) reported one week later on the purification of amorphous forms of liver concentrates.

Concurrent with the research on the active substance in liver, nutritionists (22, 57) had reported that rats required for normal growth an unidentified factor which was contained in casein, liver extracts, dried skim milk, eggs, feedstuffs and certain leafy foods. Similar studies were under investigation in poultry nutrition, and an active factor was found for chick growth and the hatchability of

[1] This review was written during December, 1951, and an effort has been made to include the fermentation literature appearing prior to that date. References for the chemistry and the assay are by no means definitive, and only casual regard has been given to the development of the nutritional and anti-anemic properties of the vitamin.

eggs. This factor, which was termed "Animal Protein Factor" or "APF", was found (30) in such products as dried skim milk, liver meal, whey solubles, meat scraps, distillers' dried solubles, fish by-products, alfalfa, cow manure and hen feces. When crystalline vitamin B_{12} became available, it was found to exhibit animal protein activity for chicks (80, 89) and to elicit growth-promoting response in rats (41). Ott et al. (89) suggested that APF and vitamin B_{12} were identical or closely related, and the designation "APF" has subsequently fallen into disuse. It should, however, be pointed out that at the present time there is doubt (28*a*, 128) that vitamin B_{12} can supplement vegetable protein so that it completely replaces animal protein from natural sources, although it is known to be a principal component of the Animal Protein Factor and to be largely responsible for the growth effect (80*g*). At the present time, emphasis is placed (10*a*, 100*d*) on the value of antibiotic-vitamin B_{12} supplements and antibiotics in the nutrition of poultry and other animals; the data obtained to date indicate that, while antibiotics stimulate growth in the presence of vitamin B_{12}, full stimulation does not occur when the vitamin is completely absent (87, 100*c*).

The first evidence that vitamin B_{12} could be a fermentation product was presented in 1948 by Stokstad et al. (133). Their bacterium (a rod-shaped, non-motile organism isolated from hen feces) produced a product which exhibited Animal Protein Factor activity in chicks and anti-anemic activity in humans. This discovery led the way towards the development of vitamin B_{12} as a fermentation product. In 1949 Petty (93) reported on the successful production of Animal Protein Factor by bacterial fermentation in aerated tanks common to industrial fermentation. Later this same year it was disclosed that both primary and secondary fermentations (with antibiotics as the primary product) had been carried out in large fermentors (83). This disclosure was followed by the announcement (26) that same year that tonnage production of Animal Protein Factor as a primary bacterial fermentation was under way. Rickes (104) showed that the product derived from fermentation and the vitamin B_{12} isolated from liver were the same. The production of vitamin B_{12} and its various complexes, in crystalline or concentrated forms and in Animal Protein Factor feed supplements, has rapidly become a major fermentation indus-

try with large markets in human nutrition and in animal feed supplements.

Isolation and purification of the vitamin B_{12} formed by the various fermentation processes will not be discussed in this review, and those interested may refer to the publications of Smith (128) and others (47*a*, 67, 74*c*, 82, 83, 83*a*, 101, 111*a*, 128*c*, 149) for information on this subject.

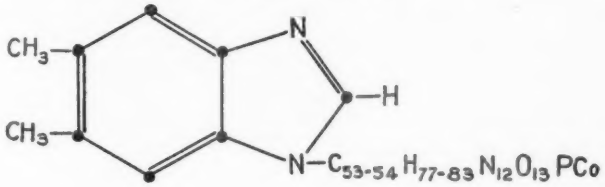
THE VITAMIN B_{12} COMPLEX

Since its isolation in crystalline form in 1948 (102), progress in the chemical structure of the vitamin B_{12} complex has been rapid, and physico-chemical studies have proved the existence of three biologically effective members of the vitamin B_{12} group. The first indication of the multiple nature of the vitamin was the report of Smith (124) on partition chromatograms of purified liver extract.

A unique feature of the structure of these naturally occurring molecules of the known B_{12} group factors is the presence of the cobalt atom (12, 103, 125); the occurrence of this atom has led to the assignment of the generic term "cobalamin" to this entire group of related compounds. The three known components differ in the nature of the radical which is coordinated to the cobalt atom. The members of the vitamin B_{12} group are comparatively large molecules with molecular weights ranging between 1440 and 1640. The structures are reportedly typified by the formulas (12):

$$C_{61-64}H_{86-92}N_{14}O_{13}PCo$$

1-alpha-D-Ribofuranosido-5,6-dimethylbenzimidazole has been characterized as an acid hydrolytic degradation product, and its structure has been established by synthesis (14). The partial and provisional formula with the dimethylbenzimidazole assumed to be terminal and linked through a nitrogen is (11, 45):



Publications from Holland (143), the United States (13, 72) and England (4) reported a cyano group attached to the cobalt atom in vitamin B_{12} (preferably termed "cyano-cobalamin"). The presence of the cyanide group in the cyano-cobalamin molecule does not confer toxicity upon it (13), and this indicates a tight binding of the cyano group within the coordination complex. This cyano group can be replaced to give various analogues, such as chloro-, sulfato- and thiocyano- compounds (19, 74), whose nomenclature has recently been clarified (40*a*). A study of the stability of cyano-cobalamin under a variety of conditions has been communicated (56, 80*c*, 80*e*, 80*f*, 107*a*).

Vitamin B_{12a} was obtained in crystalline form (71) by hydrogenation of cyano-cobalamin, and crystalline vitamin B_{12b} was isolated from fermentation broths by Pierce et al. (98). Subsequent spectroscopic (15, 145) and chromatographic (3, 145) evidence showed the probable identity of these two substances, and more recent workers concluded that they were identical (73). However, Ellis and Petrow (31*a*) believe they may not be identical in the solid state but only in solution. Kaczka et al. reported that vitamin B_{12a-b} differs from cyano-cobalamin, in the replacement of the cyano group by the hydroxyl group (13, 72), and more recent nomenclature (72) assigns the designation "hydroxo-cobalamin" to vitamin B_{12a-b}. Conversion of cyano-cobalamin to hydroxo-cobalamin with sodium bisulfite (47) or under the influence of light plus weakly acidic solution was accomplished (15, 143), as well as reversal of this reaction with cyanide (40, 72, 143, 145). Smith (126) reported the isolation of vitamin B_{12c} from fermentation broths; this compound was referred to as "factor X" and was subsequently identified (18) as B_{12c}. It has been isolated in crystalline form from fermentation liquors by Anslow et al. (3). Vitamin B_{12c} has characteristic absorption spectrum and partition behavior; its cobalt-coordinated radical is a nitrite (131). A fourth component, vitamin B_{12d}, was proposed (3), but subsequent study (128*a*, 128*b*) has shown this to be identical with hydroxo-cobalamin. All three known components of vitamin B_{12} complex are red crystalline substances, and all have been isolated in crystalline form from fermentation liquors:

cyano-cobalamin produced by *Streptomyces griseus* (104, 148) and *S. aureofaciens* (99);

hydroxo-cobalamin by *Streptomyces griseus* (47, 73, 130), *S. aureofaciens* (79, 98, 99) and *S. fradiae* (67, 86); vitamin B_{12c} by *Streptomyces griseus* (3).

These members of the vitamin B_{12} complex are believed to be present as such in the fermentation broths, since their isolation can be achieved by physical methods alone. The distinction among these different factors may be of significance in clinical treatment, as concentrates derived from various fermentations undoubtedly contain these factors in different proportions and may also contain other factors. Both cyano- and hydroxo-cobalamin, however, have been reported (6, 70) to have the same type and apparently the same degree of hemopoietic activity upon clinical test (70, 99*a*), to be equally effective for chick (16, 70, 73) and rat (73) growth, and to promote equal microbiological response (16, 59, 70, 73, 80*c*, 99*a*). Lewis et al. (78*a*, 78*b*) have isolated a vitamin B_{12}-active material, B_{12f}, differing from the known components of the complex when chromatographed and having a different absorption spectrum. This compound showed microbiological activity but possessed no growth-stimulating ability in the rat.

The possibility that additional components of the vitamin B_{12} complex remain to be isolated should not be overlooked. Woodruff and Foster (150) have reported on the occurrence, in fermentation liquors of *Clostridium tetanomorphum*, of microbiologically active substances with chromatographic values different from those of cyano- and hydroxo-cobalamin. Other workers (139*a*) have found in broths of *Serratia marcescens*, vitamin B_{12}-active substances which differed chromatographically from the known forms. The formation of pseudo-vitamin B_{12} compounds by an unidentified organism has recently been reported (96); these compounds showed microbiological activity, but they were inactive in animals. Recent investigations (96*a*) have shown the presence of three pairs of pigments in the pseudo-vitamin B_{12} series; these pairs include three cyano-cobalt pigments and the corresponding hydroxo-compounds. Two of the pairs have been obtained in crystalline form and are active as microbiological growth factors but are inactive in chicks, rats, and man; the third pair has no demonstrable biological activity. The pseudo-vitamin B_{12} compounds appear (39*c*) to differ in the occurrence of adenine instead of 5,6-dimethylbenzimidazole in the nucleotide portion of the molecule. Evidence is accumulat-

ing for various forms of bound vitamin B_{12}, probably linked with peptides or proteins (*57b*, *74d*, 140, 145). Such bound forms appear to occur, for example, in fermentation liquors, serum, liver, walls of the intestine and possibly milk (127, 142, 145). Cyano- and hydroxo-cobalamin have been found (144) to exist in the form of covalent cobaltic complexes with bonding different from that previously reported, and addition of cyanide to solutions of cyano-cobalamin has resulted in further binding to form complexes containing one or two moles of cyanide in addition to the cyano group already present in the original cyano-cobalamin (31). Thus these substances differ in the method of bonding as well as in the radicals bonded.

A United States patent (101) has been issued on "the compound vitamin B_{12}, an organic substance containing cobalt, together with carbon, nitrogen, hydrogen, oxygen, and phosphorus, said compound being a red crystalline substance soluble in water, methyl and ethyl alcohol and phenol, and insoluble in acetone, ether and chloroform, and exhibiting strong absorption maxima at about 2780 Å., 3610 Å. and 5500 Å., and an L.L.D. activity of about 11,000,000 L.L.D. units per milligram".

Recent reviews on the chemistry of vitamin B_{12} complex have been written by various investigators in the field (42, 44, 128).

METHODS OF ASSAY OF VITAMIN B_{12} COMPLEX

The possibility of a microbiological assay for the determination of vitamin B_{12} complex was first disclosed by Shorb in 1947 (115, 116) who reported that *Lactobacillus lactis* (Dorner) responded to an unidentified heat-stable factor (L.L.D.) found in liver extracts. This response was in almost linear relationship to the unit potency of the extracts used in the treatment of pernicious anemia. Since this discovery, many microbiological assays have been devised and have been widely investigated. Various media and a number of different strains and species of *Lactobacillus* have been recommended. These organisms have been employed either in turbidimetric assays, in which the amount of growth in a series of tubes containing a range of dilutions is measured; in cup-plate assays; or in titrimetric assays, in which acid production is determined. Interference can be caused in all these bacterial methods by the presence of thymidine and other purine and pyrimidine deoxyribosides which also bring about stimulation of the growth

rate of these bacteria (113, 114, 153). These deoxyribosides, unlike vitamin B_{12}, are not destroyed by alkali, and the assay sample may be re-assayed subsequent to alkali destruction of vitamin B_{12} to ascertain the response attributed to the deoxyribosides (63). These interfering factors are, however, diluted out in assay when the concentration of the vitamin B_{12} complex is sufficiently high, as in fermentation broths (120). In feedstuff materials with low concentrations of the vitamin, interference with the vitamin B_{12} assay is marked (7, 91), and differential assay techniques must be used with the bacterial assay.

When crystalline vitamin B_{12} became available, the microbiological assay of the vitamin was confirmed, and the actual incorporation of vitamin B_{12} within the cells of dependent assay organisms was established (39*a*) by radioactive tracer techniques. Crystalline cyano-cobalamin was found (102) to contain 11,000,000 L.L.D. units per milligram; the potency of all of the active members of the vitamin B_{12} complex is currently measured in terms of this factor. The fermentation figures are expressed in gamma per milliliter or per milligram, and one gamma is equivalent to 11,000 L.L.D. units.

Following the discovery of the response of *Lactobacillus lactis* (Dorner) to vitamin B_{12} complex, the use of two strains of *L. leichmanii* was simultaneously reported—one for the turbidimetric assay of the Animal Protein Factor (119), the other for the turbidimetric detection of the Anti-pernicious Anemia Factor (62). Since *L. leichmanii* requires a less complex medium and since *L. lactis* under certain conditions (117) dissociated into a form which no longer required the growth factor, *L. leichmanii* was generally adopted as the organism of choice for the turbidimetric assay of vitamin B_{12} complex (17, 21, 43*a*, 63, 90, 100*b*, 107, 120, 147) and for the titrimetric assay (80*d*, 97); use of *L. lactis* as an assay organism was restricted to the plate method (35, 36*a*, 36*b*, 46, 74*e*) adapted from the techniques employed for estimation of antibiotics and vitamins, including biotin, thiamine and riboflavin. Recent workers (32) have modified techniques employing *L. lactis* and have found this organism to be satisfactory for the turbidimetric assay. Statistical analyses have been made (17*a*, 17*b*, 83*b*) on the results obtained with the turbidimetric and titrimetric assay methods using *L. leichmannii*.

As vitamin B_{12} is predominately an intracellular product which is not excreted in quantity by the cells, it is imperative that fermentation materials be acidified or steamed for the release of growth-promoting factors (46, 77, 83, 93, 105 128*c*). Plant products have been treated with papain and takadiastase (90) for this release, animal tissues and fluids have been treated with trypsin, pepsin or pancreatin (90, 108, 110, 112, 139*a*, 139*b*, 140, 141), and an investigation of the enzymatic methods used for the liberation of vitamin B_{12} complex from animal tissues showed that short-term autoclaving was equally effective (111, 140). The practice of treating gut contents and feces during the release of vitamin B_{12} with cyanide has been recommended (28).

It was noticed in 1949 that reducing agents, such as thioglycolic acid (135), ascorbic acid (135) and cysteine (63), prevented the destruction of vitamin B_{12} activity during the necessary autoclaving of fermentation samples. Pretreatment with cyanide also prevented destruction and converted not only hydroxo-cobalamin but other naturally occurring substituted forms of the vitamin to cyano-cobalamin (121); and potassium cyanide was incorporated in the assay medium with *Lactobacillus leichmanii* (132) and with *L. lactis* (32) to permit this conversion to take place. The importance of converting naturally occurring compounds of the vitamin B_{12} complex to cyano-cobalamin (by cyanide treatment) before assay with cyano-cobalamin as standard, or of converting to hydroxo-cobalamin (by photolysis or acidification) with hydroxo-cobalamin as standard, was stressed in order that false high or low values might be eliminated (17). As work on the chemical nature of vitamin B_{12} complex continued, it became apparent that full biological activity of the modified forms depended upon their conversion before or during the assay procedure. Since the growth of *L. leichmanii* was found (17, 80*c*) to be stimulated to a greater extent when cyano-cobalamin was autoclaved, cyano-cobalamin is conceivably transformed into a new compound when autoclaved with the assay medium. This new compound is reported to have greater activity (17). The fact that cyano-cobalamin loses most of its bound cyanide when autoclaved under assay conditions (10) also indicates that a conversion takes place. Hydroxo-cobalamin and vitamin B_{12c} showed no such enhancement effect with *L. leichmanii* (17); in fact, determinations of hydroxo-cobalamin with

this organism were found to be decreased by autoclaving unless reducing agents or cyanide were present to bring about its conversion to cyano-cobalamin (59, 132).

Other bacteria which have been reported to require vitamin B_{12} for growth include *Lactobacillus acidophilus* (74*a*), *L. bifidus* (60), *L. debrueckii* (74*a*), *L. goyoni* (25), *L. helviticus* (74*a*), *Thermobacterium juhurt* (69), *T. lactis* (69) and mutants of *Escherichia coli* (37); the latter were reported to be sufficiently stable for use in assay methods (5, 20, 36*c*, 39*b*, 55*a*, 68, 145*a*). Hutner et al. (66) found that the green alga *Euglena gracilis* var. *bacillaris* exhibited a quantitative specific growth response to vitamin B_{12}, and evidence that this alga did not respond to growth factors known to be active for the Lactobacilli was also apparent in chromatographic studies (97*a*). With this organism the assay is more sensitive but requires continuous light and takes from four to seven days. However, *Euglena* remains to date the only organism of sufficient sensitivity to estimate vitamin B_{12} in body fluids (108). A strain of *Chlamydomonas* was also reported to require vitamin B_{12} (66). Lochhead and Thexton (80*a*, 80*b*) have found that the requirement for vitamin B_{12} is by no means restricted to a small number of organisms, but is shown by an important ecological group of bacteria comprising part of the indigenous microflora of the soil. These authors found that eight per cent of 534 bacterial strains isolated from the soil were dependent upon vitamin B_{12}.

In 1950 the first of a series of papers on the chemical determination of vitamin B_{12} was presented (8). The following year, recently developed analytical methods (which permitted the specific determination of one part in 100 billions of cyanide) were applied (9) to the chemical assay of cyano-cobalamin in a wide variety of substrates including aqueous extracts of fresh liver, microbial concentrates, urine and gastric juice. By this method (free or loosely bound) cyanide, cyanide liberated from cyano-cobalamin by illumination and cyanide obtained by the oxidation of thiocyanate were quantitatively separated (10). Another chemical method of analysis for vitamin B_{12} has recently been devised (108*a*, 108*b*). This determination is based on the difference between the spectrum of vitamin B_{12} and the spectrum of the solvent extraction of its dicyanide complex formed in solutions with excess cyanide; the solvent

extraction eliminates the major portion of interfering pigments generally found in fermentation broths.

The use of paper partition chromatography in the separation of various microbial growth factors has been investigated (146). A drop of the test solution is placed at one end of a paper strip, and the active factors are separated by travel of the developing agent along the strip. The strip is then placed on nutrient agar seeded with the test organism. After incubation, zones of growth of the organism are found along the strip wherever the necessary growth factors are present. The relative concentration of these factors is estimated by the size of the zones. Chromatographic separation has been applied to studies of the distinction of vitamin B_{12} complex from other materials which stimulated the growth of *Lactobacillus lactis* (36) and *L. leichmanii* (89*a*, 147), and a modified method involving test tube microbiological assay of sections of the impregnated paper strips has been reported (154). Paper strip chromatography has also been employed (36, 139, 150) to differentiate among members of the vitamin B_{12} complex.

MICROORGANISMS PRODUCING VITAMIN B_{12} COMPLEX

Organisms screened for the production of vitamin B_{12}-like activity have been isolated from soil, chicken feces and bovine feces. The first active culture was isolated from chicken feces in 1948 (133) and was subsequently identified (93) as *Flavobacterium solare*. The first active soil isolate was reported (81) in 1949, and in the same year active strains were isolated from bovine feces (93). The microorganisms which are known to produce compounds having vitamin B_{12}-like activity include Gram-positive and Gram-negative bacteria (rods and cocci), yeasts, molds and actinomycetes:

Aerobacter aerogenes (51, 128*c*)
Ashbya gossypii (83, 122, 123)
Bacillus megatherium (49, 50, 77, 88, 152)
Bacillus subtilis (51, 88, 104)
Clostridium butyricum (83, 149*a*)
Clostridium cochlearium (83, 149*a*)
Clostridium flabelliferum (83, 149*a*)
Clostridium tetanomorphum (83, 149*a*, 150)
Eremothecium sp. (83)
Eremothecium Ashbyii (149*a*)
Escherichia coli (51, 88, 117, 149*a*)
Flavobacterium acetylicum (94)

Flavobacterium acidificum (94)
Flavobacterium aquatile (94)
Flavobacterium arborescens (94)
Flavobacterium devorans (55)
Flavobacterium esteroaromaticum (94)
Flavobacterium flavescens (94)
Flavobacterium solare (93, 94)
Flavobacterium suaveolens (94)
Lactobacillus arabinosus (104)
Lactobacillus casei (117)
Mycobacterium phlei (74*b*)
Mycobacterium smegmatis (74*b*, 104)
Mycobacterium tuberculosis (74*b*, 92*a*)
Nocardia sp. (20*a*)
Propionibacterium sp. (27)
Propionibacterium freudenreichii (55*b*, 76*a*)
Propionibacterium shermanii (55*b*)
Propionibacterium zeae (55*b*)
Proteus sp. (61*a*)
Pseudomonadaceae (20*a*, 61*a*, 88)
Serratia marcescens (139*a*)
Staphylococcus aureus (51)
Streptococcus faecalis (117)
Streptomyces albidoflavus (83, 149*a*)
Streptomyces antibioticus (83, 104, 149*a*)
Streptomyces aureofaciens (98, 99, 134, 136, 138, 139)
Streptomyces colombiensis (83, 149*a*)
Streptomyces fradiae (67, 86)
Streptomyces griseus (3, 18, 47, 47*a*, 58, 74*c*, 78, 82, 83, 83*a*, 100*a*, 101, 104, 108*c*, 111*a*, 126, 127, 128*c*, 130, 138, 139, 139*a*, 148, 149*a*)
Streptomyces olivaceus (55)
Streptomyces roseochromogenus (83, 104, 149*a*)
Torula sp. (83, 149*a*)

The presence of vitamin B_{12} complex in activated sewage sludge has been disclosed (64). During the study of the oxidation of dairy wastes, these workers discovered that the mixed microflora produced significant quantities of vitamin B_{12} as detected by direct assay with *Lactobacillus leichmanii*, by alkaline destruction and by chromatographic separation. The fact that this is a large potential source of vitamin B_{12} can not be overlooked. However, the potencies obtained in sewage sludge would have to compete economically with those obtained by tank fermentation.

The most extensive search for vitamin B_{12}-producing organisms which has been reported in the literature is that conducted at the Northern Regional Research Laboratory (52). Some 5,000 organisms, including molds, yeasts, actinomycetes and bacteria, were screened for the production of vitamin B_{12}-like activity. *Flavobacterium devorans* was the most productive isolate, and no yeasts

or molds were found to be active. This group of workers later surveyed several hundred actinomycetes (55), including *Streptomyces*, which had been collected for an investigation of their antibiotic productivity (54); a large proportion of these isolates were producers of vitamin B_{12}, and yields as high as two gamma per milliliter were obtained (55). Further search through organisms collected for a survey of antibiotics at the Northern Regional Research Laboratory showed (100) many vitamin B_{12}-producers among the antibiotic-producers. Another survey of actinomycetes screened in a soybean meal medium fortified with cobalt resulted in four isolates which gave yields greater than one gamma per milliliter (109); it was suggested that greater yields of vitamin B_{12} might be expected among cultures of *Streptomyces* not characterized as *S. griseus*. In a study of approximately 150 microorganisms isolated from poultry house litter and droppings (51), *Aerobacter aerogenes* was found to be the best producer with a yield of 0.4 gamma per milliliter obtained in a medium fortified with cobalt. Using a synthetic medium with cobalt, 25 per cent of approximately 375 colonies isolated from pond mud were found (105) to produce vitamin B_{12}, as evidenced by the growth of *Euglena gracilis;* of the 92 active isolates, 88 were bacteria, two were actinomycetes and two were filamentous fungi. In addition, these authors reported activity with one pink yeast which had been isolated from fresh soil. They also tested 88 shaker flask broths (without cobalt) of actinomycetes isolated from soil; of these, 76 were stimulatory, and a maximum value of 0.5 gamma per milliliter was obtained. It is of interest that, in their study of soil extracts, 60 per cent of the colonies isolated on an acid medium at pH 5.0 stimulated growth of *E. gracilis*, and only 10 per cent of the colonies isolated on a neutral medium did so. Other investigators (118) screened 100 actinomycetes for the production of vitamin B_{12}-like activity. Their primary testing was carried out on agar plates, and the authors found no correlation either between the elaboration of red pigment in the agar and the production of vitamin B_{12} in shaker flasks or between the production of vitamin B_{12} in surface growth, as evidenced by the size of the primary screening zone, and the submerged production of the vitamin. They also screened approximately 1000 mutants which had been isolated after exposure to mutagenic agents. The maximum productivity reported in this

survey was 1.5 gamma per milliliter of vitamin B_{12}. One hundred isolates of *Nocardia,* which were selected subsequent to ultraviolet irradiation, were investigated in cobalt-containing medium (*20a*) for the production of vitamin B_{12}-active substances. The parent strain was isolated in a survey of 1213 microorganisms obtained from soils, seed washings, cow manure and poultry house litter. In this survey, 64 per cent of 537 bacteria and 66 per cent of 674 actinomycetes were capable of synthesizing vitamin B_{12}-active substances. The maximum yield obtained by these investigators was 0.4 gamma per milliliter.

While vitamin B_{12} has been isolated from antibiotic fermentation broths, including grisein (101, 104, 148, 149*a*), streptomycin (47, 83, 83*a,* 101, 108*c,* 111*a,* 130), aureomycin (79, 98, 99, 134, 136) and neomycin (67, 86), it has been shown (86) that optimum conditions for fermentation of the antibiotic are not necessarily optimum for production of vitamin B_{12}. It is of interest that in a conjunctive fermentation the trend of the curve of vitamin B_{12} production was found (108*c*) to parallel that of antibiotic production throughout the fermentation cycle.

PRODUCTION OF VITAMIN B_{12} COMPLEX

Organisms produce vitamin B_{12} complex under a wide variety of conditions, and improvements in the yield have been obtained by strain isolation, modification of the growth medium and selection of optimum conditions for the fermentation process. The greater part of the reported research has been conducted in submerged aerated culture in which the organism is grown under conditions which distribute the growth throughout the liquid medium.

The vitamin B_{12} fermentation need not, however, be aerobic (61, 83), and grain bran may be used in place of aqueous medium (83, 94). Submerged methods, with their relative ease of large-scale manipulations, are generally preferred for industrial fermentation. The reader is directed to publications of investigators in the fermentation field (33, 75, 76, 92, 95) for descriptions of equipment and procedures. In general, fermentation processes either result from a percentage conversion of the carbohydrate source by the organism into the desired products, such as solvents or acids, or represent by-products from the metabolic processes of the organism on a parts per million basis, as in the case of certain vitamins

and antibiotics. The vitamin B_{12} fermentation falls into the latter category, and the actual amount of the vitamin in the fermentation liquor or mash is only a trace when compared to the large amounts of alcohol which can be obtained in other fermentations.

Selection of high-yielding strains plays an extremely important role in the development of fermentation processes, and vitamin B_{12} is no exception, with yields increased from less than 0.1 gamma per milliliter to the two gamma per milliliter currently reported (53). The selection of cultures may be by direct isolation or by isolation subsequent to induced mutations brought about by specific chemical and physical means. These mutant cultures may show cultural and morphological differences which frequently reflect basic physiological and biochemical changes resulting in greater fermentative capacities.

Many microorganisms have a tendency to show a weakening of their ability to ferment after repeated transfers, and the conditions of sub-culture and storage of the selected strain must be carefully controlled. The number of transfers made between the master culture and the inoculum used for the production fermentor may also present a problem with some isolates, and the proper organization of seed stages may become a critical factor in the fermentation.

The temperature of any fermentation is of primary consideration, and the cultures used vary considerably in their optimum temperature requirements. The range reported for the vitamin B_{12} process extends from 24 to 30° C for fermentations with actinomycetes (48, 51, 83, 83*a*, 100*a*, 108*c*, 109, 149*a*) and up to 40° C for the bacterial fermentations (50, 74*b*, 77, 83, 94, 95, 149*a*).

The productivity of a strain may be considerably enhanced by variations in the medium in which it is propagated, and selection of suitable raw materials as nutrients for the fermenting organism has also played a significant part in the development of the vitamin B_{12} fermentation. The preferred medium in any industrial fermentation is chosen on the basis of cost considerations, availability of raw materials and ease of handling during the fermentation and recovery of the product. The recovery process is an important sequel to any fermentation, and it must be investigated jointly with the selection of cultures and media. The media used for production of vitamin B_{12} contain the usual nitrogen and carbon sources and various minerals necessary for the growth of the organisms.

Any fermentable carbohydrate or related carbonaceous material which is attacked by the specific organism employed may be used as a source of carbon. The reported carbon sources have included beet molasses (49, 50, 52, 77), cane molasses (50, 52), corn syrup (50, 83, 149*a*), dextrin (76*a*, 94), dextrose (20*a*, 50, 52, 53, 76*a*, 77, 83, 94, 100*a*, 105, 108*c*, 149*a*), fructose (50), galactose (94), glyceride oils (94), glycerol (50, 74*b*), lactose (76*a*), malt extract (94), maltose (50, 52, 77, 83, 93, 94, 149*a*), mannitol (50), mannose (94), soluble starch (50, 94), sorbitol (50, 94), sucrose (50, 51, 77, 94) and xylose (83, 149*a*). These carbon sources may be added entirely at batching or may be fed to the fermentation at intervals during the growth cycle (50, 52, 77).

The reported nitrogen sources have included ammonium nitrogen (49, 50, 52, 74*b*, 77, 83, 100*a*, 149*a*), animal stick liquor (48), asparagin (50, 74*b*), beef extract (20*a*, 58, 83, 94, 108*c*, 149*a*), blood meal (83, 149*a*), casein and enzymatic digests of casein (20*a*, 58, 76*a*, 77, 83, 94, 105, 149*a*), copra meal (61*a*), corn meal (94), corn steep liquor (48, 52, 53), cottonseed meal (61*a*, 94), distillers' solubles (48, 83, 94, 149*a*), dried yeast (1, 2, 61*a*, 83, 94, 149*a*), fish meal (83, 88, 94, 149*a*), fish press liquid (138, 139, 139*a*), fish solubles (83, 149*a*), grain alcohol stillage (123), hydrolyzed protein material (50, 83, 94), nitrates (1, 94), oats (83, 149*a*), penicillin mash filtrate extracted and desolventized (93, 94), peptone (20*a*, 52, 88, 94), protein meat and bone scraps (61*a*, 83, 149*a*), salmon waste meal (83, 149*a*), skim milk (27), soy bean meal (1, 20*a*, 48, 52, 53, 61*a*, 83, 94, 109, 149*a*), urea (50, 94), whey (27), yeast extract (20*a*, 49, 50, 51, 76*a*, 77, 83, 94, 149*a*) and yeast hydrolysate (52).

A typical aerobic bacterial fermentation of vitamin B_{12} complex is that reported by investigators at the Northern Regional Research Laboratory (52) in which the following medium was employed in their fermentors: glucose, 20 gm.; soy meal, 20 gm.; corn steep solids, 10 gm.; ammonium sulfate, 3 gm.; monopotassium phosphate, 3 gm.; magnesium sulfate, 2 gm.; cobaltous chloride, 1.0 p.p.m.; water to one liter. This medium was inoculated with five per cent of a 24-hour culture of *Flavobacterium devorans* grown in a medium containing a trace of cobalt. A temperature of 30° C was maintained throughout the fermentation. Samples of fermentation medium withdrawn from the fermentor during the course of

the fermentation showed the following pH values and concentrations of vitamin B_{12} complex as assayed by *Lactobacillus leichmanii*:

Age in hours	pH	gamma/ml. Vitamin B_{12}
0	6.6	....
16	6.6	0.085
25	6.4	0.150
40	6.3	0.204
49	7.0	0.216
63	7.5	0.440
73	8.0	0.416
87	8.5	0.576
97	8.6	0.184

The pH decreased slightly early in the fermentation cycle and then rose to alkaline levels as the fermentation approached completion. Rapid destruction of the vitamin B_{12} complex may be seen in the marked loss in potency encountered in the last sample at pH 8.6.

Other government investigators (50, 77) have reported on a bacterial fermentation of vitamin B_{12} complex with *Bacillus megatherium*. They observed that production of the vitamin lagged behind cell production, and they recommend that the culture be incubated an additional hour or two after maximum cell production occurs. The data obtained (50) follow:

Age in hours	pH	Dry weight of bacterial cells mg./ml.	gamma/ml. Vitamin B_{12}
3	6.9	1.4	0.013
4	6.5	2.8	0.027
5	6.7	5.7	0.049
6	6.7	7.0	0.056
7	6.9	13.2	0.070
8	6.7	15.4	0.115
9	6.5	22.1	0.157
10	6.6	30.0	0.335
11	....	32.5	0.500
12	6.7	39.0	0.633
13	7.3	39.5	0.775

The medium for a typical aerobic vitamin B_{12} fermentation (83), in which both streptomycin and vitamin B_{12} were formed, follows: soy bean meal, 30 gm.; dextrose, 20 gm.; distillers' solubles, 7.5 gm.; sodium chloride, 2.5 gm.; cobaltous ion, 1 p.p.m.; water to 1 liter. This medium was inoculated with ten per cent by volume of

a vegetative culture of a streptomycin-producing strain of *Streptomyces griseus*. After a 48-hour fermentation at 27° C, assay of the final acidified filtered broth showed 0.245 gamma per milliliter of vitamin B_{12} with *Lactobacillis lactis* (Dorner). Anaerobic fermentation with a non-streptomycin-producing strain of *S. griseus* was carried out (83) for 72 hours at 28° C in a medium composed of dried yeast, 20 gm.; cobaltous ion, 10 p.p.m; water to one liter. An assay of 0.437 gamma per milliliter was obtained in the acidified filtered broth. Anaerobic flask fermentations were conducted (83) with *Clostridium tetanomorphum* at 35° C without agitation in the following medium: brain heart infusion, 37 gm.; agar, 3.7 gm.; cobaltous ion, 2 p.p.m; water to one liter. A maximum yield of 0.454 gamma per milliliter of vitamin B_{12} as assayed by *L. lactis* (Dorner) was obtained in the acidified filtered broth.

As has been previously mentioned, all three known members of the vitamin B_{12} group have been isolated from fermentation liquors. The culture medium and the microorganism are influential in determining the concentration and the proportion of the growth factors present in the broth. Woodruff and Foster (150) have presented evidence to show the range of results obtained from the growth of *Streptomyces griseus* in three media (whole protein, protein hydrolysate, and synthetic) and from the growth of *Clostridium tetanomorphum* in two media. Not only was the recovery of vitamin B_{12} activity different from the various media, but the distribution of hydroxo- and cyano-cobalamin in the broths showed marked variation, as evidenced by microbiological activity and paper strip chromatography. These authors also reported that other microbiologically active substances differing from hydroxo- and cyano-cobalamin in chromatographic values are present in the fermentation broths of *C. tetanomorphum;* these constituents may represent nearly all or less than ten per cent of the total active factors, and their relative concentration depends upon the medium employed.

The use of possible precursors or intermediates in the production of vitamin B_{12} complex by microorganisms has been investigated to a limited extent concurrent with advances in discerning the chemistry of this vitamin group. While progress has been made in the addition of cobalt to the fermentation, other lines of approach have not been so fruitful. The possibility exists, however, that the

search may be as rewarding as the addition of the phenylacetyl derivatives to the fermentation of penicillin (29, 85).

Wood and Hendlin (149*a*) in 1948 found cobalt to be an extremely important factor in the fermentation of vitamin B_{12}, and they filed application for a United States patent at that time. Their work clearly established the fact that the addition of cobalt in concentrations of about 0.1 to 20 parts per million of the nutrient medium markedly increased fermentation yields. Its use as an additive in the fermentation medium was studied in detail by Hendlin and Ruger (58) with *S. griseus;* they concluded that cobalt, a structural component of the vitamin B_{12} molecule, became the limiting factor (in their specific medium) for the microbial synthesis of the vitamin. Other investigators (20*a*, 50, 51, 52, 74*b*, 77, 83, 86, 100*a*, 105, 109, 138, 139) have adopted addition of cobalt to the initial fermentation medium as standard procedure. Increased yields of vitamin B_{12} were reported (48) to be associated with intermittent feeding of five p.p.m. cobalt to a synthetic medium inoculated with an unidentified form of *Streptomyces,* and addition of cobalt to the inoculum medium as well as to the fermentation medium was also communicated (52, 77). It is of interest that Chaiet et al. (24) reported biosynthesis of radioactive vitamin B_{12} by *S. griseus* with cobalt^{60} in the fermentation medium. Other investigators (39) showed that the fecal B_{12} content of rats could be increased by raising the amount of Co^{60} in the diets, and cobalt has been shown to be of definite benefit for chickens, mice and rats kept on rations low in vitamin B_{12} (10*a*, 67*a*).

The compound 1,2-dimethyl-4,5-diaminobenzene has been identified as part of a dimethylbenzimidazole riboside which occurs in acid hydrolysates of vitamin B_{12} (14). Wooley (151) found that this dimethyldiamine was able to fulfill the vitamin B_{12} requirements of lactic acid bacteria and algae. Further work by this author (152) indicates that 1,2-dimethyl-4,5-diaminobenzene could be a precursor for vitamin B_{12}; approximately twice as much vitamin B_{12} was produced by *Bacillus megatherium* when this postulated precursor was added to the medium. The dimethyldiamine was also found (43) to possess vitamin B_{12} activity for the rat. This compound, however, was subsequently reported by Hendlin and Soars (59*a*) to be not only devoid of biological activity for *Lactobacillus lactis* (Dorner) but actually to be inhibitory for this

organism as well as for *L. leichmanii* and *Euglena gracilis*. In the presence of amounts of vitamin B_{12} sufficient to initiate cell growth, these authors found that the toxicity of the dimethyldiamine for *L. lactis* was reduced and that this toxicity was competitively and specifically antagonized by vitamin B_{12}. They also reported that various substituted anilines possessed vitamin B_{12} activity for *L. leichmanii*, provided half maximal levels of vitamin B_{12} were present in the medium. These authors believed, however, that this effect was not due to microbial synthesis by the assay organism, since other workers (23) had detected no synthesis of vitamin B_{12} when substituted analogues were added to broths inoculated with *L. lactis*. They hypothesize that the toxicity of the dimethyldiamine inhibits the growth of vitamin B_{12}-requiring organisms within the intestinal tract, thus making more of the vitamin available to the host. Several structural analogues of 1,2-dimethyl-4,5-diaminobenzene were found (152*a*) to cause marked reductions in the synthesis of vitamin B_{12} by *B. megatherium* when used in concentrations not inhibitory to growth. The inhibition of synthesis was seen with those analogues which were non-competitive antagonists to 1,2-dimethyl-4,5-diaminobenzene as well as with those which were competitive antimetabolites. *Serratia marcescens* produced vitamin B_{12}-like activity in medium containing unnamed degradation products of the vitamin, but showed no activity when grown in medium without vitamin B_{12} or its degraded fractions (139*a*). Brief mention has been made (108*c*) of the apparent effect of inoculum medium containing precursors upon fermentation yields of vitamin B_{12} with *Streptomyces griseus*. Inoculum grown in meat extract medium was reported to result in greater productivity than inoculum grown in soybean-yeast medium. The authors expressed the opinion that precursors structurally similar to vitamin B_{12} occurred in meat extract and were responsible for this increased production.

Addition of vitamins, including para-aminobenzoic acid, nicotinic acid and pantothenic acid, to the fermentation medium with *Flavobacterium devorans* has recently been reported (52) to result in greater yields of vitamin B_{12}. The specific effect of para-aminobenzoic acid upon the synthesis of vitamin B_{12} by mutants of *Escherichia coli* was studied in detail (38). A definite sparing effect of vitamin B_{12} on the para-aminobenzoic acid requirements of the

mutant strains was found, and the relative requirement was equally compatible with the function of para-aminobenzoic acid as a catalyst or as a structural precursor in the synthesis of vitamin B_{12}. Recent work, however, has shown that pseudovitamins B_{12} and B_{12b}, which lack the benzene ring, exert the same sparing action as vitamin B_{12}. Thus the suggestion of para-aminobenzoic acid as a structural precursor does not seem plausible at this time (38*a*). The finding that inclusion of riboflavin in vitamin B_{12}-deficient diets could overcome avitaminosis in rats led to the interpretation that riboflavin may be utilized by microorganisms in the intestinal tract and converted to vitamin B_{12}-active material (57*a*).

OCCURRENCE OF VITAMIN B_{12} COMPLEX IN PLANT TISSUES

At this time the only primary source of vitamin B_{12} in nature appears to be the metabolic activity of microorganisms and algae; there is no convincing evidence for its elaboration in tissues of higher plants or animals. Traces of the vitamin in soil (34, 105) and in water (105, 106) probably arise from microbial and algal synthesis. The vitamin B_{12} detected in the roots of higher plants (105, 106) could be accounted for by microbial origin in the soil. Reports on the presence of traces of vitamin B_{12} in alfalfa are conflicting but may be partially explained by recent developments in differential assay techniques and paper-partition chromatography. By the use of these new methods, 85 to 89 per cent of the apparent vitamin B_{12} activity of alfalfa has been attributed to other growth factors (7, 96). Such materials as lawn grass, soy bean sprouts, barley, potatoes, beans, cabbage and green peas have been found devoid of vitamin B_{12} activity as determined by the rat assay (78, 155).

In early studies on the occurrence of vitamin B_{12} in feedstuffs and in various plant tissues, the values obtained microbiologically and those obtained by animal bioassay did not satisfactorily correlate. Part of this difficulty can be ascribed to the interference of thymidine and other deoxyribosides in the microbiological assay, and part may be ascribed to incomplete liberation of the active factor from the tissues. Using the alkali treatment of correcting for the effect of deoxyribosides (63), a number of products of plant origin were studied for their corrected and uncorrected content of vitamin B_{12}, and the following concentrations were noted (91):

Food product	Vitamin B_{12} uncorrected (gamma per gram)	Vitamin B_{12} corrected (gamma per gram)
Wheat	.007	.001
Soybean meal	.010	.002
Yellow corn	.005	0
Oats	.010	.003
Alfalfa meal (dried)	.027	.003
Brewers' yeast (dried)	.017	.001
Distillers' solubles	.008	.0005

Since these feedstuff products were obtained from natural sources and since microorganisms were associated with the root systems of the plants in the soil, no conclusions can be drawn in regard to the ability of the plants to synthesize vitamin B_{12}.

Use of *Euglena gracilis* as an assay organism avoids the interfering substances encountered with bacteria and permits detection of lower concentrations of vitamin B_{12}; this alga has been employed by Robbins et al. (105, 106) for determination of vitamin B_{12} in various naturally occurring substances and in plant tissues. These authors found (105) that gelysate, trypticase, neopeptone and peptone contained 0.050 to 0.150 gamma vitamin B_{12} per gram; malt extract and yeast contained 0.002 to 0.004 gamma per gram; corn steep liquor contained 0.004 to 0.010 gamma per gram; pepsin and malt diastase contained 0.020 gamma per gram; and lots of commercial agar ranged up to 0.003 gamma per gram. They could detect no activity in barley, wheat germ or yellow corn meal. These authors (105) investigated the vitamin B_{12} content of the roots of beets, string beans, broccoli, Brussels sprouts, cabbage, celery, chicory, endive, kale, kohlrabi, horse-radish, leek, lettuce, perennial onion, green pepper, red pepper, radish, rhubarb, perpetual spinach, strawberry, New Zealand spinach, Swiss chard, tomato and turnip; all gave positive tests for the vitamin, with quantities ranging from 0.004 to 0.010 gamma per gram of fresh material. Negative results were obtained with carrot, Chinese cabbage, parsnip and sweet potato. The results with leaves, except in the case of radish, were negative. The fruits of avocado, banana, coconut, grape, peas, green pepper, persimmon and string bean were also negative; no activity was detected in germinated seeds of barley, corn, peas, radish, rutabaga, soybeans, string beans, Swiss chard and wheat. It is apparent that positive results were obtained for the majority of roots tested and that leaves, fruits and seeds were generally negative. The vitamin B_{12} content of the roots of a

given species showed considerable variation. The authors comment that no evidence was found for the synthesis of this vitamin within the plants themselves.

Tomato plants and cuttings were also investigated (106) under non-sterile conditions. The roots of field-grown tomatoes were found to contain up to 15 gamma of vitamin B_{12} per gram of fresh weight, whereas the developed roots of cuttings contained 0.78 gamma per gram.

Aqueous extracts of species of green, red and brown algae obtained from natural sources were assayed with *Euglena gracilis* for vitamin B_{12} content. The values obtained (106) ranged from 0.0001 gamma to 0.1000 gamma per gram of dried alga; the authors, however, point out that more complete methods of extraction would probably have given higher results. Of considerable interest are their values obtained with pure cultures of three blue-green algae grown in an inorganic medium. While their data obtained with other algae may, as they state, have been affected by possible derivation from associated microbial production of vitamin B_{12}, the blue-green algae were actual producers of the vitamin; potencies as high as 0.64 gamma per gram were obtained with aqueous extracts of the blue-green algae. The algae tested included the following:

Blue-green algae	
Plectonema Nostocorum	0.07 gamma per gram
Calothrix parietina	0.64 gamma per gram
Aphanizomenon flos-aquae	0.28 gamma per gram
Diplocystis aeruginosa	0.24 gamma per gram
Marine algae	
Ceramium rubrum *Champia parvula* *Chondria tenuissima* *Enteromorpha intestinalis* *Lomentaria Baileyana*	0.05 to 0.10 gamma per gram
Agardhiella tenera *Chondrus crispus* *Polysiphonia variegata* *Ulva Lactuca*	0.01 to 0.045 gamma per gram
Ascophyllum nodosum *Chorda Filum* *Chordaria flagelliformis* *Fucus vesiculosus* *Laminaria Agardhii* *Mesogloia divaricata* *Sargassum Filipendula*	less than 0.01 gamma per gram

SUMMARY

During the four years which have passed since the microbiological fermentation of vitamin B_{12} complex was first accomplished, major developments have occurred, and the production of vitamin B_{12} and its various complexes has become a major fermentation industry with substantial markets in human and animal nutrition. Factors which have contributed to the highly successful developmental work on the production of this vitamin include the isolation of cultures, the selection of media, a study of the optimum conditions of temperature, aeration and agitation necessary for large-scale fermentation, and the establishment of suitable recovery processes for the fermentation broths. The natural occurrence of this vitamin appears to be attributable to microorganisms and algae instead of higher plants or animals.

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ROOTROTS OF CEREALS. II[1]

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INTRODUCTION

In the original review (45) an attempt was made to cover most of the pertinent literature, from which was drawn information concerning the major types of root diseases in cereals. These types were then discussed in regard to their economic importance and the main research trends pertaining to each. Perhaps the most impressive feature brought out in the review was the close association between soil conditions and cropping practices and the incidence of root maladies. Subsequent reports in the literature on rootrots reveals, as might be expected, a considerable concentration by workers upon various aspects of the relationship between soil factors and root diseases. An overall view of the literature for the past ten years shows that root maladies have received considerable attention. In Europe and the British Isles, take-all, a serious root disease of wheat and barley, continues to receive much attention. In North America research on take-all continues, but the common rootrots that attack all cereals appear to be emerging as a major challenge. There are large cereal-growing areas where, no doubt, researches on diseases are being conducted but from which, because of the late war and the unsettled period following, few reports are received. A perusal of the literature on rootrots of cereals, appearing through the period under review, shows no important new development in research trends but a considerable exploration of problems opened up earlier. However, there has appeared one new disease of oats, Helminthosporium blight, that should be included in any discussion on rootrots. For a comprehensive survey of root disease fungi in general, the reader is referred to the treatise by Garrett (17) published in 1944.

In the present discussion of rootrots of cereals, the arrangement will be, for the most part, in accordance with types of disease, followed by brief concluding remarks.

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TAKE-ALL

Take-all, caused by *Ophiobolus graminis,* must be classed as a major rootrot of cereals. Under some conditions it is extremely destructive, but it can be effectively controlled in most cases. It will always receive much attention because of its occasional sudden appearance and extensive damage in wheat and barley crops. Winter (69), following a period of rather intensive investigation on the influence of soil structure, temperature and moisture on the infection of wheat by *O. graminis,* published the results of some work on biotic factors. He concluded that accumulation of carbon dioxide, as originally suggested by Garrett (10), is not a limiting factor in the growth of the hyphae of this fungus in natural soils. On the other hand, his work indicated the presence of inhibiting substances in the soil which are partly destroyed by heat. Destruction of these substances would explain the better growth of *O. graminis* in partially sterilized soils. A comprehensive piece of work on partial sterilization of soil and its influence on *O. graminis* was later reported by Winter (70). From this investigation the activity of soil micro-organisms antagonistic to *O. graminis* was well established as the principal inhibitory source, although complicating factors may arise from various soil types. In applying these findings for the control of take-all under field conditions, it was suggested that the best approach is to modify the soil by addition of organic materials; these materials favour growth and multiplication of the antagonists. Winter (71, 72) further investigated the part played by soil micro-organisms as antagonists of *O. graminis.* He observed the presence of a great abundance of actinomycetes after a period of two weeks in partially sterilized soil, a phenomenon that did not occur in natural soil. These actinomycetes destroyed the hyphae of *O. graminis* by direct parasitism or by secretion of toxins. In natural soils, bacteria, actinomycetes and fungi react to the presence of the hyphae of *O. graminis,* resulting occasionally in a beneficial as well as a harmful influence on the pathogen. *O. graminis* responded favorably to the addition of nutrients, such as peptone, glucose and biomalt, under sterile conditions, but under non-sterile conditions the influence of antibiosis produced variable results. Some studies were made also on the influence of the rhizosphere of different plants on the growth of *O. graminis.* Slagg and Fellows (49) contributed to the study of

the relationship between *O. graminis* and a large number of soil fungi. Particular attention was given to the influence of by-products produced by the test fungi, in both laboratory and field experiments. Substances having an inhibitory effect on *O. graminis* were found rather frequently, but some stimulatory by-products were demonstrated as well. The culture medium had a definite influence in determining the nature of the by-product. In continuing the earlier studies of Henry reported in the first review, Ludwig and Henry (33) conducted some experiments with sterilized soil that was subsequently recontaminated. They determined the influence of this soil on the infection of wheat by *O. graminis*. Micro-organisms increased rapidly in the recontaminated soil and suppressed infection. Considerable significance was attached to the fact that one of the fungi that increased rapidly was *Trichoderma viride*, a fungus known to be highly antagonistic to *O. graminis*.

Garrett, in England, continued his studies on soil conditions in relation to the take-all disease of wheat. He found (11) that the supply of available nitrogen in the soil was closely related to the survival of the mycelium of *O. graminis*. The experimental work consisted of studying the survival of the fungus in infected wheat straws buried in the soil. Longevity was greater in soils well supplied with nitrogen. It was thought that the decrease in survival under conditions of microbiological activity was caused by a rapid consumption of food material in this activity, as well as by *O. graminis* itself. The available food supply is dependent upon available nitrogen. Later, Garrett (12) showed that *O. graminis* can under certain conditions utilize nitrate nitrogen. That the nitrogen relationship might have some application in the control of take-all was demonstrated in some interesting experiments by Garrett (15, 16). He found that infected pieces of wheat straw retained the pathogen much better in fallow soil than in soil under crop. It was concluded that in the fallow soil much of the nitrogen produced by the activity of soil organisms was utilized by *O. graminis*, thus prolonging the survival of this pathogen; on the other hand, in soil supporting a crop, the available nitrogen, or much of it, was taken up by the growing plants. Therefore it would be expected that *O. graminis* will disappear more rapidly in soil under a non-susceptible crop than in fallow soil because of the depletion of nitrogen reserves and the lack of a congenial host to support survival. His

hypothesis was supported by an observation that in certain areas where barley, a susceptible crop, is grown year after year on the same land, very little take-all occurs if barley crops are undersown with trefoil which makes luxuriant growth after the barley has been cut. This late heavy growth of the trefoil reduces the available nitrogen in the soil. Further information (22) on the control of take-all in wheat was obtained from experiments conducted at Woburn in which the available supply of nitrogen was affected by cultural and cropping practices. Fertilizer experiments were conducted by Garrett (13, 18) with wheat in pots and with barley in the field, to determine the influence of plant nutrition on the severity of take-all infections. Although there were infection differences as various combinations of nitrogen, potash and phosphate were tried, it appears from the results that a good balanced nutrition offers the best protection to the host. The importance of maintaining soil fertility, particularly the balance between available phosphorus and nitrate nitrogen, in reducing take-all damage was clearly shown in the work of Stumbo, Gainey and Clark (51); it would appear from the results of their investigation that under certain conditions soil fertility is of greater importance than the soil microflora in the control of take-all.

The report by Turner (53) of a variety of *Ophiobolus graminis* that attacks oats, causing a disease of the take-all type, is of considerable importance. Heretofore, oats, because of their resistance to *O. graminis,* have always been recommended as a control measure to follow a wheat crop attacked by take-all. The new variety, *O. graminis* Sacc. var. *avenae,* was found chiefly on oats in Wales. A form of take-all of oats has been reported also from Australia and Denmark. Later, Garrett and Dennis (21) reported the occurrence of *O. graminis* Sacc. var. *avenae* in Scotland and England.

A considerable amount of work on take-all has been done in Australia. White (62) studied the sexuality of *O. graminis* and found the fungus to be homothallic. In later studies (64) on the genetics of this pathogen, single ascospore isolates were used to determine segregation for pathogenicity and culture colour. The results of a preliminary field experiment with single spore isolates was reported (65); the isolates showed differences in pathogenicity. White (63) studied the growth factor requirements for *O. graminis* and showed that biotin and thiamin were very impor-

tant. Furthermore, it was shown that wheat roots and straw contain nutritional factors and that extracts of these contain, as well, certain growth factors. Peptone and asparagine were found to be important nutrients for the growth of this fungus. He discussed these various factors in relation to parasitism and later (66) showed that, in making isolations from root lesions, the use of a biotin-free medium would separate other invaders from *O. graminis.* In continuing his physiological studies on *O. graminis,* White (67) conducted experiments to determine its carbon and nitrogen requirements. He found that a wide range of organic compounds is utilized as sources of carbon, and of organic and inorganic compounds as sources of nitrogen. Carbon in the carbohydrate form with nitrogen from asparagine and peptone permitted the greatest assimilation. White (68) reported on greenhouse and field experiments with wheat that showed the importance of a healthy root system, especially the crown roots, in reducing injury by take-all. Continuous cropping, weed competition and low soil moisture resulting in low fertility, predisposed the crop to greater injury because diseased roots could not be readily replaced. A dry top soil was particularly unfavourable. The earlier work by Ludbrook (32), in which use was made of the root-amputation technique, showed also a relationship between a dry top soil and poor crown root development. Under these conditions, when the wheat plants are supported mainly by the seminal roots, attacks by the take-all fungus are very injurious.

A wheat disease, new to India, was diagnosed as take-all and reported by Padwick (39) in 1940.

Control of take-all is accomplished by crop rotation, cultural methods and maintenance of good soil fertility and structure. These conclusions, especially the importance of rotations, were arrived at by all investigators who studied this malady. Garrett (14) gives a good summary of control measures. Varietal resistance has played no important role in controlling take-all of wheat; all varieties of bread wheats have been susceptible. It is interesting, therefore, to note a report by Ciferri (7) whose observations indicated that *Triticum dicoccum* and *T. spelta* were somewhat resistant and *T. monococcum* practically immune to *O. graminis.* Common wheats were susceptible. If such resistance is confirmed by later work, the next step should be a breeding programme with the

objective of transferring this resistance to *Triticum vulgare* lines and varieties.

COMMON ROOTROT

Common rootrot is the term employed in this review to designate the type of necrosis of cereal crowns and roots caused by *Helminthosporium sativum* and *Fusarium* spp. Other pathogens may cause similar symptoms and could be discussed also under common rootrot. In the previous review the economic importance of common rootrot was discussed. Additional reports on losses in western Canada from this complex malady were made by Sallans and Ledingham (43), Machacek (34) and Sallans (42). The last mentioned report was a comprehensive study of the interrelation of many factors, including seasonal rainfall, air temperatures and rootrots, in their effects on wheat yields.

There has been continued interest in the antagonism or antibiosis between soil and other micro-organisms and the common rootrot pathogens. Christensen and Davies (6) showed that *Bacillus mesentericus,* a bacterium prevalent in soil, could, when grown on artificial media, produce a substance that was toxic to *H. sativum.* The toxic action caused abnormal growth in this pathogen and at certain concentrations produced variants. Some of the variants were considered to be mutations; these mutants appeared to have a greater tolerance for the toxic substance. Antagonism between *H. sativum* and *F. culmorum,* two root disease pathogens, was observed by Ledingham (29) in inoculation tests on wheat. Johnston and Greaney (27) studied the pathogenicity to wheat of a large number of *Fusarium* isolates. Besides the usual observations on soil moisture and temperature in respect to virulence, they noted that *Trichoderma lignorum* and *Pyronema confluens,* two common soil inhabitants, suppressed the virulence of *F. culmorum.* In the same series of tests the virulence of this pathogen was not influenced by *Penicillium intricatum* or *Aspergillus flavipes.* Sanford (44), in 1946, published a general review of the situation in regard to the influence of the microflora associated with various crops and soil amendments on soil-borne diseases, including common rootrot of cereals. Some interesting data were obtained by Tyner (54, 55) in his work on soil amendments, particularly composts, in relation to rootrots of cereals. In his earlier studies, composts of soil with

oat straw were more effective in reducing rootrot of wheat than composts with wheat or barley straw. The later study showed that any of the cereal straws added to the soil greatly increased the soil microflora. However, if the soil contains a good amount of natural inoculum of *H. sativum* or *Ophiobolus graminis,* wheat plants grown in such soil, regardless of the increased microflora, show a fair amount of infection. In fact the presence of wheat roots appeared to cause an increase in numbers of some of the soil fungi. These investigations indicate, once again, the complexity of the interactions between soil flora and rootrot pathogens. The explanation for some of these phenomena may well lie along the approach explored by Garrett (15, 16) and White (63, 67) in their studies on the nutritional and growth factor requirements of the pathogen. The occurrence of a bacterial flora, antibiotic to *H. sativum,* on wheat straw and seed was reported by Simmonds (46). Later work in the same laboratory by Ledingham, Sallans and Simmonds (30) showed the importance of the antibiotic flora on wheat seed in inoculation tests and tests for varietal resistance. In their experiments a true measure of inherent resistance could be obtained only by first removing the antibiotic flora. The importance of the antibiotic flora in the soil in controlling rootrot of barley caused by *H. sativum* was demonstrated by Anwar (2). He found one isolate of *Bacillus subtilus* to be particularly active in suppressing *H. sativum* in inoculation experiments. The widespread occurrence of *H. sativum* in wheat fields of western Canada was reported by Simmonds, Sallans and Ledingham (48). Their observations indicated the prevalence of inoculum, mainly conidia, on or near the soil surface where it may be washed about by seasonal rainfalls or floods. Experiments showed that the microflora on stubble or in the surface soil could suppress *H. sativum,* and, in turn, this antibiotic flora, which was considered to be largely bacterial, might be suppressed or killed by sunshine. Laboratory experiments showed that, with mixed cultures of bacteria and conidia of *H. sativum,* proper exposures to ultra-violet light killed the bacteria without injuring the conidia.

Unlike the take-all disease, which usually can be controlled by crop rotation, common rootrot is difficult to combat; in fact, specific and practicable control measures are not known. Many of the recent contributions therefore have been studies towards control

methods. There is general agreement that good farming practices in regard to rotations, rate and depth of seeding, and fertility, for the region concerned are valuable in reducing losses from common rootrot. Greaney (25) reviewed some of the pertinent literature and reported the results of his experiments on the influence of time, rate and depth of seeding wheat and the incidence of common root-rot. He concluded that losses could be reduced by seeding spring wheat early, when soil temperatures are low, and not too thickly or deeply. In Saskatchewan evidence was obtained (48) indicating that conidia and mycelial fragments of *H. sativum* accumulated on the soil surface or on surface debris. Primary infections of wheat and barley arose from these sources. Preliminary studies on the inactivation of this surface inoculum by plowing, instead of the usual shallow surface cultivation, were encouraging.

Workers on root diseases quite naturally turned their attention to the selection or development of resistant varieties, an approach that proved successful in the control of many other plant diseases. Pisarev and Malinovskaya (40) studied rootrot caused by *Fusarium* spp., particularly *F. avenaceum, F. culmorum* and some species of the Elegans section. Some resistant wheat varieties and hybrids were found by these investigators in their plant breeding work. Studies in western Canada by Tyner and Broadfoot (57) on the resistance of a large number of spring wheat varieties to various rootrots, including common rootrot, were inconclusive. The work was conducted under field conditions. Varieties fell into certain groups in respect to degree of resistance, but the authors emphasized that factors, such as soil conditions and stage of maturity, must be taken into account. Laboratory methods for testing seedlings of wheat varieties were described by Simmonds and Sallans (47). They grew the seedlings in either test tubes or Petri dishes, suitably fitted with blotter or filter paper, and inoculated them with *H. sativum*. The tests showed that seedlings of the varieties Marquis, Thatcher, Red Bobs and Apex have more resistance than seedlings of Mindum, Regent, Reward, Pelissier and Renown.

One of the several difficulties encountered in making tests for resistance or pathogenicity with common rootrot pathogens, such as *Fusarium* spp., has been the great variability in the fungus. This problem has been mentioned by many workers. Recently

Oswald (37) studied the *Fusarium* spp. involved in a cereal root-rot complex. He found, within the scope of the original species concept as propounded by Wollenweber and Reinking (73), a great variation in both cultural characteristics and pathogenicity among the isolates studied; he concluded that the classification suggested by Snyder and Hansen (50) is more satisfactory. In regard to another and perhaps the most important common rootrot pathogen, *H. sativum,* the recent paper by Tinline (52), reporting the discovery of the perfect stage of this fungus, should prove valuable. With this information the pathologist has the advantage of a better understanding of the variability of the isolates of *H. sativum,* and this may facilitate testing for varietal resistance. It has been known to all workers on rootrots of cereals that seed treatments have only limited application as a means of control. When the pathogens, however, are carried on or in the seed in sufficient quantity, seed treatments have proved to be valuable, a fact well demonstrated in investigations conducted by Machacek and Greaney (35). Common rootrot is the only major group of cereal rootrots in which the pathogens may be seed-borne.

ROOT DISEASE OF CEREALS CAUSED BY PYTHIUM SPP.

Browning rootrot of cereals, mainly wheat, was at one time of great economic importance in western Canada. There has been, however, a noticeable decline in its importance during recent years. In 1940 Vanterpool (58) reported further results of his field and laboratory studies. These results support his earlier findings that browning rootrot of wheat caused by *Pythium* spp. can be controlled by application of phosphatic fertilizers or barnyard manure to infested soils. Recently Vanterpool (59) has analysed the various factors concerned in the decline of browning rootrot of wheat. He concludes that the decrease in disease can be attributed to recent changes in cropping practices, viz., widespread use of phosphatic fertilizers, incorporation into the soil of large quantities of crop residues left after modern combine harvesting, and use of Thatcher wheat, a somewhat more resistant variety, in place of Marquis. The prevalence and virulence of *Pythium arrhenomanes* Drechsl., one of the more important pathogens of browning rootrot of cereals and grasses, was shown by the work of Vanterpool and Sprague (60). Their investigation revealed the common occurrence of this

pathogen on a large number of grasses commonly found in the cereal areas of North Dakota and adjacent areas of Canada. A root disease of oats caused by *Pythium debaryanum* Hesse was reported by Welch (61). It caused serious trouble in Iowa in the years 1938 and 1942. The pathogen is an active root invader, particularly causing necrosis of the rootlets throughout the seedling stage. The loss of roots results in poor growth, with a yellowing and dying of the basal leaves. It was found that nutritional deficiencies were involved which could be corrected under field conditions by proper use of fertilizers. There was no marked resistance to *P. debaryanum* in any of the numerous oat varieties tested. In regard to resistance to the attacks of *Pythium* spp., the work of Elliott (9) with corn is of interest. She worked with *P. arrhenomanes* Drechsl. and tested it against 12 inbred lines of dent corn, with results indicating resistant, susceptible and intermediate lines.

OTHER TYPES OF CEREAL ROOTROTS

Several other types of rootrot of cereals were discussed in the earlier review, and some attention must be given here to recent literature concerning them. The eye-spot lodging disease of wheat, caused by *Cercosporella herpotrichioides*, was reported in a brief note by Gorter (24) as causing rather serious trouble in experimental plots in South Africa. The distribution of this malady was further extended by first reports from Australia (1) and Canada (8). From time to time reports appear describing injury to the crown and roots of cereal plants by *Rhizoctonia solani* Kuhn. In most cases these attacks have been of minor importance. Blair (3) studied this fungus in regard to its pathogenicity to wheat and also in regard to its behaviour and survival in the soil. He worked with isolates obtained from wheat in England and Canada. In pathogenicity tests, all isolates were more virulent in natural than in sterilized soil. Parasitism, however, was reduced by addition of cellulosic organic materials to unsterilized soil, indicating, it would appear, microbial antagonism and competition. The isolates could be divided into two groups: those from England parasitized the roots mainly, whereas the Canadian isolates attacked the shoot. The Rossi-Cholodny soil-plate technique was found satisfactory for measuring the growth of *R. solani* in different soils (4). A brief report on crown and rootrot of cereals caused by *R. solani* in the spring wheat area of South Dakota was published by Bruehl (5).

The fungus was not considered to be a serious root disease pathogen. Glynne and Richie (23) reported the presence in England and Wales of a disease of wheat (sharp eye spot) caused by *R. solani.*

A serious Helminthosporium blight of oats, which in the later stages of the plant's growth is manifested by severe stem and root-rot, was reported by Meehan and Murphy (36). After careful study they concluded that a species of *Helminthosporium* was involved. It was described and named by them as *H. victoriae.* The name was suggested by the observation that oat varieties and selections having resistance to crown rust (*Puccinia coronata avenae*) derived from the variety Victoria were susceptible to this fungus. This relationship was definite and consistent. Helminthosporium blight was particularly destructive in Iowa, Illinois, Indiana and New York in 1946 (31) because of the large acreage sown to oat varieties carrying the Victoria type of resistance to crown rust. The connection, however, between susceptibility to Helminthosporium blight and the variety Victoria made it comparatively easy for the plant breeder to select or develop varieties without this weakness. According to Kingsolver (28), the use of resistant varieties, supplemented by seed treatments, will give good control of Helminthosporium blight.

GENERAL

An ecological classification of soil fungi with special reference to root-inhabiting fungi and soil-inhabiting fungi was outlined and discussed by Garrett (19). The root-inhabiting group includes the specialized parasites and the mycorrhizas. The soil-inhabiting group includes the unspecialized parasites and the obligate soil saprophytes. The parasitic characteristic of the root-inhabiting fungi has been gained through a long period of host-parasite relationship while their saprophytic qualities declined. Survival of root-inhabiting fungi is dependent upon the presence of the host. On the other hand, soil-inhabiting fungi can survive indefinitely as soil saprophytes. The microbiological interactions concerned in both the parasitic and saprophytic phases of root-inhabiting fungi are fully discussed. Garrett's analytical approach to the complexity of factors involved in dealing with root diseases should help in clarifying the situation in respect to any component in the extensive group of root maladies, for instance, rootrots of cereals. In a later

paper on the subject of ecological groups of soil fungi, Garrett (20) emphasizes the substrate relationship. He mentions, as examples of groups based upon substrate connections, the following categories: saprophytic sugar fungi, root-inhabiting fungi, lignin-decomposing fungi, coprophilous fungi and predaceous fungi.

Tyner and Broadfoot (56) obtained extracts toxic to wheat seedlings from cultures of *Helminthosporium sativum* and *Fusarium culmorum* grown in nutrient solutions fortified by 0.5% sucrose. The extracts did not lose their toxicity with sterilization.

An interesting study of a relationship between insect damage and rootrot of cereals and grasses was reported by Hanson, Milliron and Christensen (26). According to their findings, certain species of billbugs, especially *Calendra parvula,* attack the crowns, lower internodes and roots, and the injured tissues are soon colonized by root disease fungi. Extensive damage to cereals and grasses sometimes results from this combined invasion.

A preliminary study of rootrots of cereals in Switzerland by Zogg (74) showed that many species of fungi are associated with diseased roots. Pathogenicity tests were conducted with mixed inoculum, and it was found, for instance, that, when *Ophiobolus graminis* and *Ascochyta graminicola* were mixed, the infection of wheat by the former was greatly increased. Similarly, heavy infections of wheat resulted from mixtures of *Fusarium oxysporum* var. *aurantiacum* and *F. orthoceras* var. *longius.*

A general survey of cereal rootrots in California was published recently by Oswald (38).

Studies of root development in wheat, including root amputation experiments, mentioned in the author's first review, as a means of aiding in the interpretation of rootrot injury, were extended in an investigation by Sallans (41). It was found that established roots contribute independently of each other to the final yield of grain. The primary seminal root was the most valuable individual root in this respect. The contributions of the crown roots, although individually small, were in the aggregate greater than those of the seminal roots.

SUMMARY

The literature on rootrots of cereals that has appeared since the first review reveals a continued interest in these maladies. There have been no major changes in research trends. There have been,

however, notable elaborations of work previously initiated, in many cases with ingeniously devised techniques. Advances have been made in studies on the relationship between root disease organisms and soil and other micro-organisms. Much information has been added to our knowledge of the take-all rootrot by intensive research in Germany, England and Australia. In North America it appears that common rootrot may now be classed as a major research challenge. Browning rootrot of wheat, once of great economic importance in Canada, has declined with the introduction of better farming practices and the application of control methods developed after years of research. Helminthosporium blight has appeared as a serious stem, crown and rootrot of oats. There are several types of cereal rootrots considered of minor importance at present, but these must be kept under surveillance. Notable progress has been made in fundamental studies on the perplexing variability of the pathogens *Helminthosporium sativum* and *Fusarium* spp.

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